β-Lactam antibiotics offer neuroprotection by increasing glutamate transporter expression

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Glutamate is the principal excitatory neurotransmitter in the nervous system. Inactivation of synaptic glutamate is handled by the glutamate transporter GLT1 (also known as EAAT2; refs 1, 2), the physiologically dominant astroglial protein. In spite of its critical importance in normal and abnormal synaptic activity, no practical pharmaceutical can positively modulate this protein. Animal studies show that the protein is important for normal excitatory synaptic transmission, while its dysfunction is implicated in acute and chronic neurological disorders, including amyotrophic lateral sclerosis (ALS)[3], stroke[4], brain tumours[5] and epilepsy[6]. Using a blinded screen of 1,040 FDA-approved drugs and nutritionals, we discovered that many β-lactam antibiotics are potent stimulators of GLT1 expression. Furthermore, this action appears to be mediated through increased transcription of the *GLT1* gene[7]. β-Lactams and various semi-synthetic derivatives are potent antibiotics that act to inhibit bacterial synthetic pathways[8]. When delivered to animals, the β-lactam ceftriaxone increased both brain expression of GLT1 and its biochemical and functional activity. Glutamate transporters are important in preventing glutamate neurotoxicity[1,9–11]. Ceftriaxone was neuroprotective *in vitro* when used in models of ischaemic injury and motor neuron degeneration, both based in part on glutamate toxicity[11]. When used in an animal model of the fatal disease ALS, the drug delayed loss of neurons and muscle strength, and increased mouse survival. Thus these studies provide a class of potential neurotherapeutics that act to modulate the expression of glutamate neurotransmitter transporters via gene activation.

To identify compounds capable of increasing rodent GLT1 expression, a structurally diverse library of 1,040 FDA-approved drugs and nutritionals were individually added to organotypic spinal cord slice cultures prepared from postnatal day 9 rats (Fig. 1a). This approach mimics the cellular metabolism and cell–cell interactions present *in vivo*. All assays were conducted in a blinded fashion, and each drug (100 μM, added biweekly) was studied in duplicate or triplicate (10–15 tissue samples per drug). After 5–7 days of drug treatment, tissue was harvested and immunoblotted for expression for GLT1 protein using GLT1 anti-peptide antibodies (Fig. 1b). Dibutyryl cyclic AMP, a *GLT1* gene activator, served as a positive control (Fig. 1b), while 0.1% DMSO controlled for drug solubilizer. Approximately 50–60 drugs per week were investigated. GLT1 protein was analysed by semiquantitative, semi-automated densitometry. Replicate variability was less than 10%. Over 20 compounds were capable of increasing GLT1 protein expression by more than twofold compared to untreated controls (Fig. 1c). Analysis of the top 2% of all hits revealed that a single class of compounds, β-lactam antibiotics, was overly represented. Fifteen different β-lactam antibiotics, including penicillin and its derivatives, as well as cephalosporin antibiotics, were highly active in stimulating GLT1 protein expression (Fig. 1d). Increased expression could be seen as early as 48 h after drug treatment. Validation of the most active drugs was confirmed by repeat treatment of spinal cord cultures with 10–100 μM drug. The EC_{50} for increasing GLT1 expression by a representative cephalosporin, ceftriaxone, was 3.5 μM (Fig. 1e), which is comparable to the known central nervous system (CNS) levels attainable with therapy for meningitis (0.3–6 μM)[12,13]. Non-β-lactam antibiotics included in the screen had no effect on GLT1 protein expression, including kanamycin, fluconazole, minocycline, polymyxin and doxycycline.

To better understand the mechanism of action, the effect of the drugs on the GLT1 promoter was examined in cell lines from

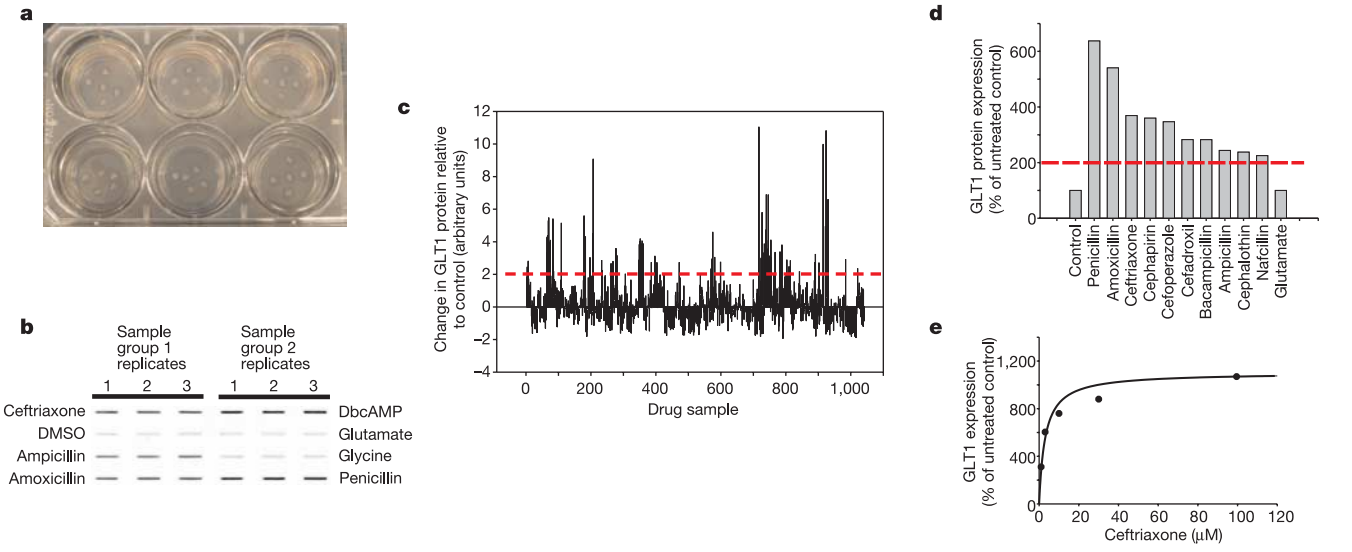

Figure 1 Screen of 1,040 FDA-approved drugs reveals β-lactam antibiotics as inducers of GLT1 protein expression. **a**, Rodent lumbar spinal cord cultures. **b**, Sample raw data slot blot of GLT1 protein in triplicate, including untreated tissue control, dibutyryl cyclic AMP positive control (dbcAMP), DMSO drug vehicle control, and various drugs (all shown here at 10 μM). **c**, Screening results for 1,040 sample compounds. Bar height reflects increased GLT1 protein expression relative to vehicle-treated controls. **d**, β-Lactam antibiotics were highly represented among the most potent compounds. **e**, Dose response analysis for ceftriaxone, revealing EC_{50} of 3.5 μM for GLT1 expression.

astrocytes and non-neuronal tissues. A 2.5-kilobase (kb) fragment of the human GLT1 promoter linked to firefly luciferase was transfected into human fetal astrocytes[7], and used to screen the active compounds identified above. Similar results were also obtained using stable cell lines of human fetal astrocytes or COS7 cells transfected with a 2.7-kb GLT1 promoter fragment linked to both enhanced green fluorescent protein (eGFP) complementary DNA and firefly luciferase cDNA. As shown in Fig. 2a, the human GLT1 promoter fragment was significantly activated by ceftriaxone, amoxicillin and dibutyryl cyclic AMP, but not by the antibiotic vancomycin, amino acids glutamate and glycine, or the vehicle, DMSO. These effects were dose dependent, seen as early as 48 h after drug administration, and persisted for at least 7 days *in vitro* (Fig. 2a). Additional analysis of various cephalosporins (10 μM) and β-lactams revealed prominent activity among the various agents (Fig. 2b), although the parent structure, cephalosporin C, was inactive in astroglial cell lines. No β-lactams were found that inhibited promoter activation.

As these compounds were capable of activating the promoter at concentrations known to be attainable in brain after parenteral administration (for example, 10–150 μM)[14], we further explored the *in vivo* biological activity of ceftriaxone in normal rats. After five to seven days of ceftriaxone therapy (200 mg per kg, i.p. daily, $n = 5$), animals were killed and brain tissue collected. Antibiotic treatment led to a threefold increase in GLT1 protein expression, and active splice variant GLT1b (ref. 15), as determined by semiquantitative immunoblots from hippocampus and spinal cord (Fig. 3a, b). This increase was persistent, and could also be observed after 3 months of treatment ($n = 10$). Conversely, the other molecular subtypes of glutamate transporters, including the astroglial protein GLAST and the neuronal glutamate transporters EAAC1 and EAAT4, were unchanged after ceftriaxone administration (Fig. 3a, b).

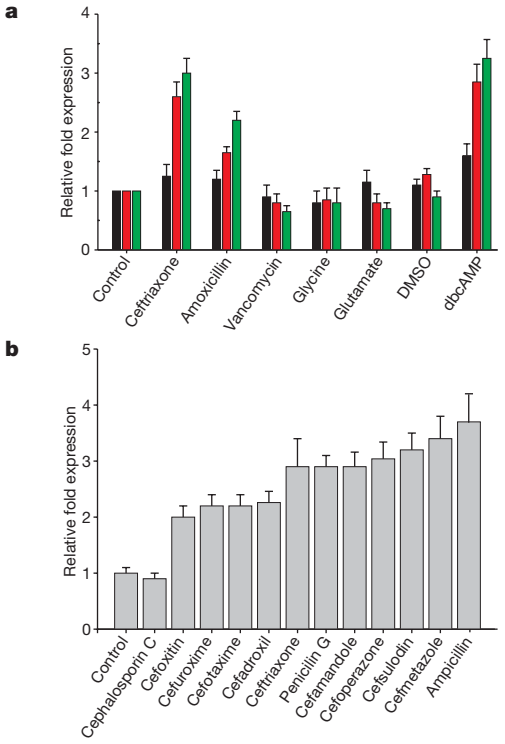

Figure 2 Promoter reporter analysis. β-Lactams activate human GLT1 promoter. **a**, In human fetal astrocytes transfected with the GLT1 promoter/luciferase reporter, β-lactam antibiotics at 0.1 μM (black), 1 μM (red) and 10 μM (green), markedly activate the GLT1 promoter in a dose dependent manner, while controls such as glutamate and glycine have no effect. Dibutyryl cyclic AMP is a known GLT1 promoter activator. **b**, Closer analysis of cephalosporin antibiotics reveals consistent activation (10 μM) by many, but not all, structural variants, while vancomycin had no effect. Data shown as mean+s.e.m.

GLT1 promoter activation was also observed *in vivo* (Fig. 3c, left panel). Chronic treatment of GLT1-BAC-eGFP promoter reporter mice with ceftriaxone produced an obvious increase in reporter expression in astroglial soma and processes throughout the hippocampal CA1 neuropil (Fig. 3c, right panel). Notably, in this brain region neuronal expression of the gene was not induced by drug (Fig. 3c, right panel). The effects of ceftriaxone appeared to be relatively specific, as the constitutive proteins actin (Fig. 3a) and superoxide dismutase 1 (SOD1, not shown), neuronal specific proteins neurofilament L and synaptophysin, and the astroglial protein glial fibrillary acid protein (GFAP), were unaffected by ceftriaxone therapy. Treatment with non-β-lactam antibiotics including vancomycin and minocycline had no effect on brain GLT1 levels.

Glutamate transporters are preferentially localized to astroglial membranes, although in some cases, increased protein expression is not always mirrored by concomitant membrane localization and functional activity[16]. However, cephalosporin therapy did increase biochemical glutamate transport, as measured by L-[^{3}H]glutamate uptake into cortical membrane (Fig. 3d) or spinal cord (not shown) homogenates prepared from adult animals treated intraperitoneally for 7 days with drug. Similarly, after 7-day treatment, ceftriaxone increased GLT1-mediated L-[^{3}H]-glutamate transport in a dose dependent fashion in cultured spinal cord slices (Fig. 3d). The increase in cell surface GLT1 was confirmed with cell membrane impermeant biotinylation reagent (Fig. 3e). Biotinylated GLT1 was increased on plasma membranes from mixed cortical neuron/astroglial cultures treated for 7 days with ceftriaxone. Finally, glutamate-transporter-associated currents tended to be larger in hippocampal astrocytes following 4–7 days of treatment of postnatal rat pups with ceftriaxone (Supplementary Data). Thus, *in vitro* and *in vivo* administration of ceftriaxone led to a threefold increase in protein levels and a comparable increase in GLT-1 specific biochemical and electrophysiological transport. Penicillin treatment also increased biochemical transport (Fig. 3d), although its brain penetration is less, presumably accounting for the lower level of activity. Vancomycin was inactive in these functional assays.

Glutamate receptor antagonism has been extensively explored in acute and chronic neuroprotection, but no therapies exist to modulate glutamate-mediated injury via transporters. Genetic overexpression of transporters in transgenic mice and in engineered cell lines suggest that increasing the density of transporter in astroglia can be neuroprotective[17]. The level of neuroprotection may depend on the magnitude of overexpression. To determine if β-lactam antibiotics, ceftriaxone in particular, could be neuroprotective, we tested the compound in a series of *in vitro* and *in vivo* models. Treatment of cultured neurons with a low oxygen, low glucose condition, known as oxygen glucose deprivation (OGD), models the neuronal injury that can occur in ischaemic injury. In this model, one hour of OGD was lethal to cultured neurons, with toxicity known to involve excess glutamate[18]. However, when cultures are preconditioned 24 h before the lethal condition with transient OGD (5 min), there is a dramatic and robust resistance of neurons to cell death. This neuroprotection, referred to as ischaemic pre-conditioning, is due in part to increased expression of GLT1 (ref. 18), although some studies suggest these transporters could contribute to ischaemic injury[2]. As shown in Fig. 4a, baseline neuronal death in the cultures was 14% (no treatment column, NT). Ceftriaxone (1 μM), when added for 48 h to cultures, did not increase the baseline cell death (NT+ceftriaxone), but increased GLT1 protein levels (>25%; not shown) and transport. Cultures subject to 1 h OGD, without preconditioning, increased neuronal death to 50%. Ischaemic preconditioning OGD (5 min) applied 24 h before a one-hour OGD prevented neuronal injury. Importantly, 1 μM ceftriaxone (or the β-lactam

cefuroxime, not shown), when added 48 h before 1 h OGD, was also protective, reducing the percentage of neuronal cell death from 50% to 20%—similar to ischaemic tolerance neuroprotection. Thus β-lactam pre-treatment appeared to prevent neuronal death in ischaemic tolerance.

Chronic blockade of glutamate transport in spinal cord organotypic cultures, with the non-specific transporter inhibitor *threo*-β-hydroxyaspartate (THA) or DL-*threo*-β-benzyloxyaspartate (TBOA)[11] leads to chronic increase in extracellular glutamate and subsequent slow death of motor neurons. To determine if ceftriaxone-induced GLT1 overexpression could be neuroprotective, we examined motor neuron degeneration in the organotypic spinal cord model. Organotypic cultures were prepared from lumbar spinal cords of 8–9-day-old rodent pups[11]. No drugs were added for the first 7 days following culture preparation. Then ceftriaxone (1–100 μM) was added with media changes, and after 7 more days, THA or TBOA were added at a concentration of 100 μM, which produces chronic death of motor neurons. After 2–4 weeks, cultures were immunostained for neurofilament to quantify large ventral horn motor neurons. As shown in Fig. 4b, ceftriaxone treatment prevented motor neuron loss in a dose dependent manner. Similar neuroprotective results were seen with penicillin (not shown). As an additional control, organotypic spinal cord cultures prepared from GLT1-null mice were not protected from THA toxicity by ceftriaxone pre-treatment (Fig. 4b). Vancomycin was not protective.

To determine if ceftriaxone could alter neurodegeneration in a disease model that involves altered expression of glutamate transporters, we treated G93A SOD1 mice with drug. Studies have documented a contributory role for excess glutamate in this model, including neuroprotection by glutamate receptor blockade[11,19–21]. Modest GLT1 overexpression can alter disease progression[17]. Guo *et al*[17] reported that a 1.5–2.3 fold increase in N-myc labelled human GLT1 expression in G93A SOD1 mice delayed disease onset as measured by grip strength (~14 days), but had no effect on other onset parameters such as weight loss and paralysis (3 days), and had no effect on survival. Initiating drug treatment in this animal model around the time of clinical disease onset at, for example, loss of strength, most closely matches the use of human therapy, and could be more therapeutically relevant[22]. G93A SOD1 mice were treated daily with ceftriaxone (200 mg kg^{-1} i.p.) starting at 12 weeks of age—approximately the time of clinical disease onset. Drug-treated animals ($n = 20$) and saline-injected controls ($n = 20$) were monitored daily for survival, and weekly for grip strength and body weight[22,23]. As shown in Fig. 4c, d, ceftriaxone treatment significantly delayed loss of muscle strength and body weight. This effect was observed within 7 days after treatment, and persisted for 4–6 weeks. By 19 weeks of age, the strength preservation was lost. In a similar manner, the drug also increased overall survival of the mice by 10 days (ceftriaxone treated, 132 ± 2 days (all data with errors show mean ± s.e.m.); saline control, 122 ± 2 days; log rank, $\chi^2 = 7.8$, $P > 0.005$; Wilcoxon $\chi^2 = 7.5$, $P > 0.006$) (Fig. 4e). This effect is typical of drugs given relatively late in the life of G93A SOD1 mice, when the first clinical signs of disease are evident, and thus even a small effect may have clinical significance. When the same dose of drug was administered somewhat earlier, at 6 weeks of age, survival was also increased (ceftriaxone treated, 135 ± 2 days, $n = 20$; saline treated 122 ± 1.9 days, $n = 20$), although not significantly better than late delivery at 90 days of age. The lack of greater efficacy when given earlier would be consistent with the observation that the loss of GLT1 expression does not begin to occur until around 90 days in this model.

To determine if ceftriaxone altered cellular neurodegeneration *in vivo*, G93A mice were treated with ceftriaxone starting at 70 days of age. Two weeks of drug therapy lead to a significant prevention of motor neuron loss (Fig. 4h, i) and reduction of hypercellular gliosis compared to saline-treated control G93A mice. GLT1 expression decreases around the onset of clinical disease[24], yet ceftriaxone administration was able to increase endogenous GLT1 expression significantly in spinal cords from the chronically treated mice (Fig. 4f–h). The neuroprotection seen in this study was not likely to be due to the normal antibiotic properties of the drug, because ALS mice are not septic and do not have lung infections at

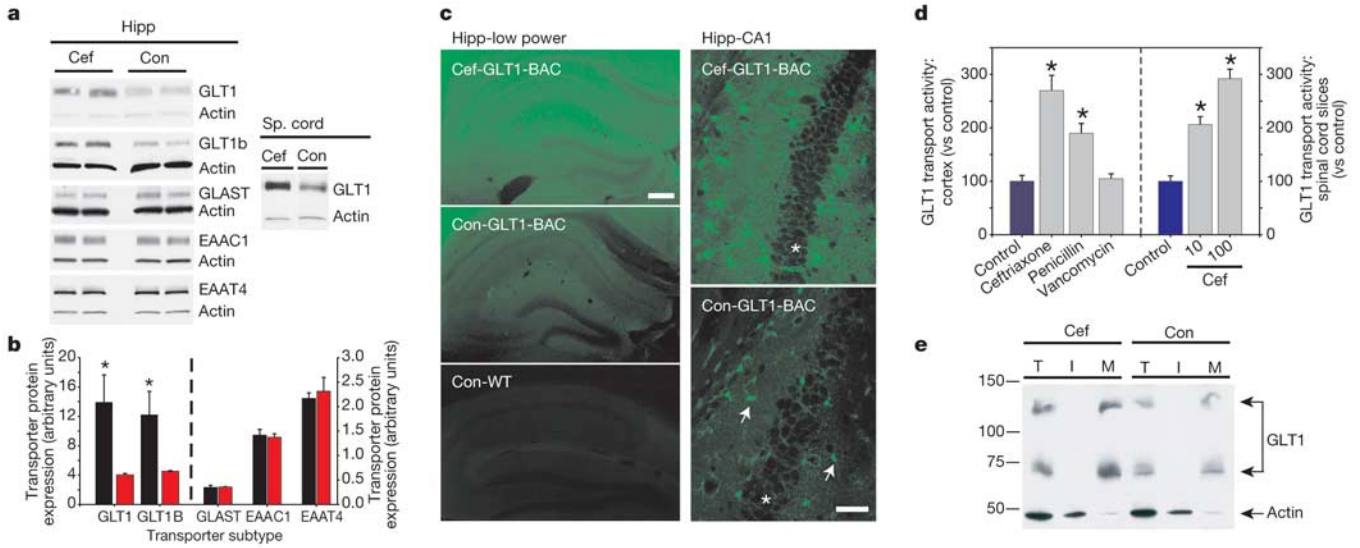

Figure 3 β-Lactam induces transporter promoter activation and protein expression *in vivo*. **a**, **b**, Ceftriaxone (black bar) induces expression of GLT1 and GLT1b protein, in hippocampus (Hipp) and spinal cord (sp. cord) (**a**, western blot) compared to saline control (**a**, control (con); **b**, red bar). Expression of the glutamate transporters GLAST, EAAC1 and EAAT4 were unaffected. **c**, Increased *in vivo* activation of the GLT1 promoter (**c**, left panels, low power light microscopy; scale bar, 200 μm), using GLT1 BAC-eGFP promoter reporter mice, in hippocampal CA1 astrocytes (asterisks; **c**, right panel, confocal microscopy; scale bar, 50 μm) and neuropil but not CA1 neurons (arrows) from drug-treated mice, compared to untreated control mice. **d**, Ceftriaxone and penicillin administration increased ^{3}H-glutamate transport in cortex homogenates from drug-treated mice (left panel) and treated spinal cord cultures (right panel). *$P < 0.05$ compared to untreated control. **e**, Immunoblots of total (T), intracellular (I) and biotinylated fractions (M) of mixed neuron/glial cortical cultures treated for 7 days with ceftriaxone (cef, 100 μM). Molecular weight markers in kDa. In **b**, **d**, data are mean+s.e.m.

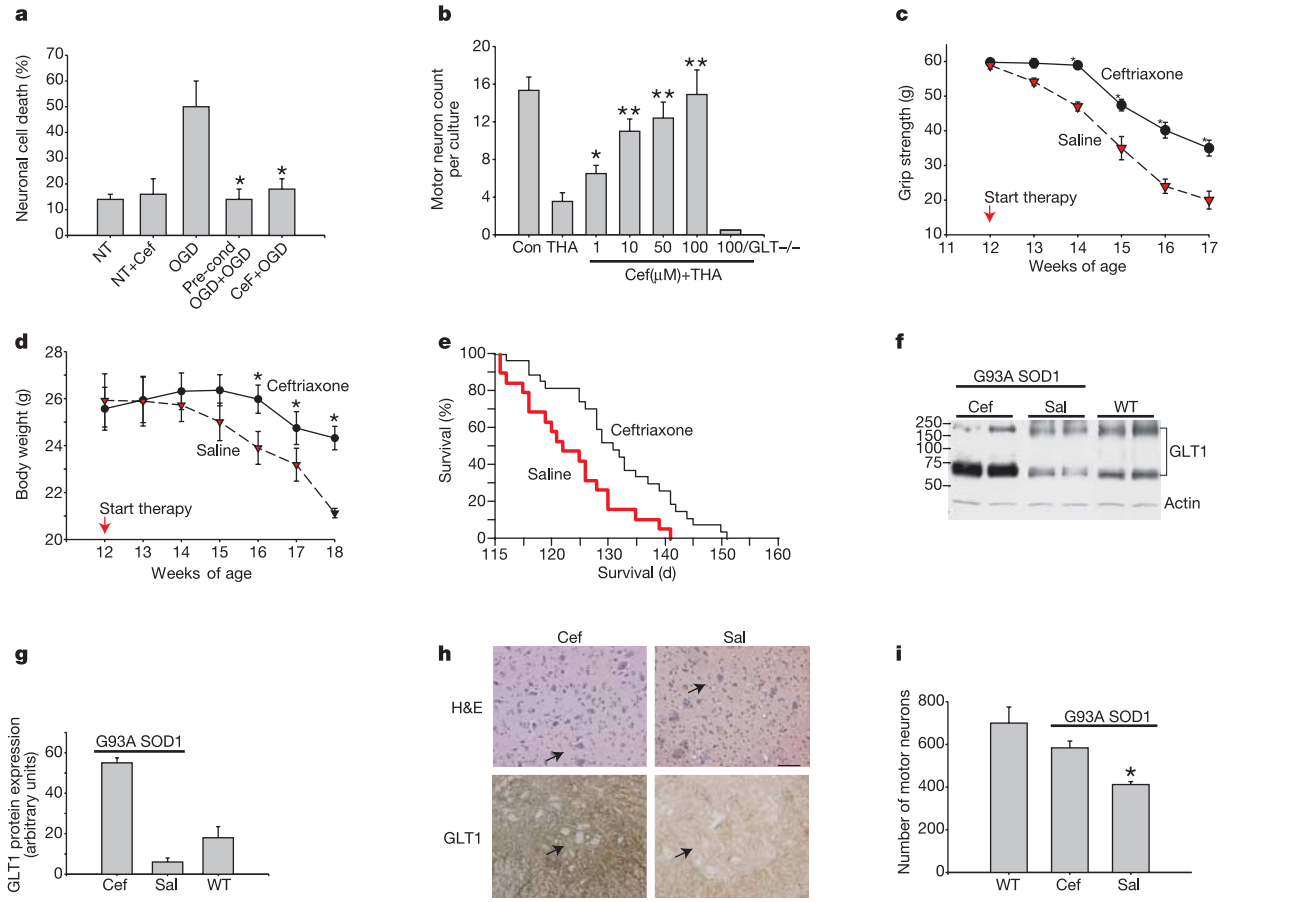

Figure 4 *In vitro* and *in vivo* neuroprotection by ceftriaxone. **a**, Oxygen glucose deprivation (OGD) of cultured cortical neurons was neurotoxic, but OGD preconditioning or ceftriaxone pre-treatment (1 μM) was protective compared to no treatment (NT). **b**, Ceftriaxone (Cef) treatment of spinal cord cultures prevented *threo*-hydroxyaspartate (THA)-induced motor neuron loss but not in GLT1-null mouse (GLT1 − /−) tissue. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus untreated control. **c**–**e**, In G93A SOD1 ALS mice, ceftriaxone initiated at disease onset (red) delayed loss of muscle strength (**c**) and body weight (**d**) compared to saline treatment (black); ceftriaxone initiated at disease onset also increased survival (**e**). Spinal cord GLT1 protein levels (**f**, **g**) and tissue expression (**h**) were markedly elevated in ceftriaxone (Cef)-treated ALS mice compared to saline (Sal)-treated ALS mice and untreated wild-type (WT) mice. Molecular weight markers in kDa. Two weeks of drug treatment (Cef) delayed loss of lumbar spinal motor neurons (**h**, **i**) compared to saline treatment in haematoxylin and eosin (H&E) stained tissue. Scale bar, 50 μm. For panels **c**, **d** and **e**, $n = 20$ saline, $n = 20$ ceftriaxone group. $^{*}P < 0.05$ versus ceftriaxone. Data in **a**, **b**, **g**, **i**, are mean + s.e.m.; data in **c**, **d**, are mean ± s.e.m.

12–16 weeks of age—when prominent muscle strength effects were seen. In addition, the use of other CNS-penetrating antibiotics when given at this late stage (12 weeks old) do not prevent loss of muscle strength (for example, minocycline; L.I.B. and J.D.R., unpublished observations).

β-Lactam antibiotics, first identified with the discovery of penicillin in 1928, are now the most widely used antibiotics, and are one of the most important modern pharmaceuticals[8]. Notably, they have no substantial toxic CNS actions at normal antibacterial doses. Our studies document a new property of these antibiotics, and demonstrate that β-lactams can activate the gene for a neurotransmitter transporter. This is, to our knowledge, the first evidence of stimulatory pharmaceutical modulation of the glutamate transporter, and provides a new pathway for drug discovery and manipulation of glutamate transmission in disease. The mechanism of this overexpression appears to be activation of the genetic promoter for GLT1, although the pathway for promoter activation is as yet unknown. □

Methods

Screening assay and protein expression

Organotypic cultures were prepared from postnatal day 9 rat lumbar spinal cords[11]. Slice cultures were maintained on Millicell-CM 30-mm inserts (5 slices per insert; Millipore, PICM 03050) in 35-mm six-well plates (Falcon no. 3046) containing 1 ml growth media, without antibiotics, and maintained in a humidified atmosphere of 5% CO_2. After 7 days *in vitro*, cultures were treated with the NINDS Custom Collection (MicroSource Discovery) drugs at a concentration of 100 μM for 7 days. Media and drugs were changed biweekly. GLT1 was quantified by slot blot (5 μg protein per slot). Protein concentration in tissue sonicates was determined using Coomassie Plus protein assay (Pierce no. 1856210). GLT1, GLAST, EAAC1 and EAAT4 protein were detected using primary rabbit polyclonal rat anti-carboxy-terminal GLT-1 antibody followed by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate (Pierce no. 34080) detection (BioRad VersaDoc, Quantity One Discovery Series software,v4.3.0)). Twenty-one compounds were selected for retesting at 10–100 μM to confirm hits (>300% of control). These compounds were also screened in a six-point dilution series with a maximum concentration of 300 μM. These dilutions were created from freshly prepared 10 mM stocks in DMSO. Concentrations required to achieve 50% of the maximally achievable effect for each compound (EC_{50}) were calculated using SigmaPlot (Ver 9; Systat).

Human GLT1 promoter reporter assay

GLT1 promoter activity was studied in normal human fetal astrocytes seeded at 1×10^5 cells per 35-mm plate. Twenty-four hours after seeding, cells received the indicated compound at a final concentration of 1–10 μM or were left untreated (control). Forty-eight hours later, the cells were transfected (calcium phosphate precipitation method[7]) with a pGL3/GLT1 luciferase reporter construct (5 μg) plus a pSVβ-galactosidase construct (1 μg). In some cases, human fetal astrocytes or COS7 cell lines transfected with the GLT1 promoter (2.7 kb) luciferase/eGFP construct were used. After an additional 48 h, cell lysates were prepared and luciferase activity was determined using the Luciferase Assay System Kit (Promega, E1501) and luminescence determined using a luminometer (Turner Designs, TD20/20)[7]. Data presented is the average of three independent plates ± s.d.

GLT1 activity and immunoblotting

Levels of GLT1 protein were quantified by immunoblots[3]. Functional glutamate transport was measured by accumulation of ^{3}H-glutamate in spinal cord slice or crude cortical synaptosomal membranes[25]. Measurement of total glutamate uptake actually reflects the combined physiological activity of all transporter subtypes. GLT1 protein is uniquely sensitive to transport inhibition by dihydrokainate (DHK). To estimate the contribution of GLT1 to transport, aliquots of tissue homogenates were also incubated with 300 μM DHK. Non-specific uptake was determined in the presence of 300 μM *threo*-β-hydroxyaspartate (THA), at 0 °C and in sodium-free homogenates.

Generation of GLT1 BAC eGFP transgenic mouse

The BAC transgenic mice were generated as described previously[26] with a shuttle vector provided by N. Heintz. The BAC clone included approximately 45 kb upstream of the first GLT1 exon, the full GLT1 coding region (123 kb) and 24 kb downstream of the last exon. EGFP cDNA was inserted into the GLT1 start codon.

Oxygen glucose deprivation/ischaemic preconditioning

Primary cortical mixed neuronal-glial cell cultures were prepared from rodent fetal cortex (gestation day 14–16 CD1 mice) using the paradigm of ischaemic preconditioning[18].

Motor neuron toxicity

Neuroprotection in spinal cord organotypic cultures prepared from postnatal day 8–9 wild-type rat or GLT1-null mouse tissue was performed as described previously[11]. Ceftriaxone was added for 5–7 days before the addition of 100 μM THA or DL-*threo*-β-benzyloxyaspartate, (TBOA) 100 μM. Surviving motor neurons were counted 2–3 weeks later by staining for phosphorylated neurofilaments (SMI-32).

G93A SOD1 mouse—disease onset and survival

Male transgenic mice expressing the human G93A SOD1 (B6SJL-TgN(SOD1-G93A)1Gur, high expressor) were bred with background-matched B6SJL wild-type females (Jackson Laboratories). The progeny were genotyped and used for subsequent studies. Experiments were conducted at Psychogenics (Hawthorne, New York) in accordance with protocols approved by the Johns Hopkins Animal Care and Use Committee. Mice were assessed by daily observation for survival, and by weekly weighing and testing of grip strength starting at 12 weeks of age[22,23]. All experiments were performed blinded with coded syringes for injection.

Histology and motor neuron counts

Mice were perfused via cardiac infusion with 4% buffered paraformaldehyde and spinal cord post fixed with the same solution. The lumbar enlargement was collected, paraffin embedded, and serially sectioned at 14 μm, for a total of 140 sections. Every seventh section was stained with haematoxylin and eosin, and examined at 20 × for motor neuron identification and counting[22]. Images were acquired using the Zeiss LSM 510 Meta confocal microscope (argon laser setting at 488 nm) with the operator blinded to treatment groups. All images were captured with the same gain, offset, pinhole diameter (2.53 Airy units), and scan speed (12.8 μs with scan averaging set to 2). Z-series images were collected at 1.03 μm intervals.

Statistics

Quantitative differences between *in vitro* and *in vivo* drug effects were analysed by analysis of variance (ANOVA) or Students *t*-test. Survival analysis was performed by Kaplan-Meier analysis. Software for statistics included Statview, and JMP 5.1 (SAS Software).

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Nucleolar proteome dynamics

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The nucleolus is a key organelle that coordinates the synthesis and assembly of ribosomal subunits and forms in the nucleus around the repeated ribosomal gene clusters. Because the production of ribosomes is a major metabolic activity, the function of the nucleolus is tightly linked to cell growth and proliferation, and recent data suggest that the nucleolus also plays an important role in cell-cycle regulation, senescence and stress responses[1–4]. Here, using mass-spectrometry-based organellar proteomics and stable isotope labelling[5], we perform a quantitative analysis of the proteome of human nucleoli. *In vivo* fluorescent imaging techniques are directly compared to endogenous protein changes measured by proteomics. We characterize the flux of 489 endogenous nucleolar proteins in response to three different metabolic inhibitors that each affect nucleolar morphology.